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Molecular design of recombinant scFv antibodies for site-specific photocoupling to β-cyclodextrin in solution and onto solid support

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ABSTRACT

The ability to design and tailor-make antibodies to meet the biophysical demands required by the vast range of current and future antibody-based applications within biotechnology and biomedicine will be essential. In this proof-of-concept study, we have for the first time tailored human recombinant scFv antibodies for site-specific photocoupling through the use of an unnatural amino acid (UAA) and the dock'n'flash technology. In more detail, we have successfully explored the possibility to expand the genetic code of *E. coli* and introduced the photoreactive UAA *p*-benzoyl-L-phenylalanine (*p*Bpa), and showed that the mutated scFv antibody could be expressed in *E. coli* with retained structural and functional properties, as well as binding affinity. The *p*Bpa group was then used for affinity capture of the mutated antibody by β-cyclodextrin (β-CD), which provided the hydrogen atoms to be abstracted in the subsequent photocoupling process upon irradiation at 365 nm. The results showed that the *p*Bpa mutated antibody could be site-specifically photocoupled to free and surface (array) immobilized β-CD. Taken together, this paves the way for novel means of tailoring recombinant scFv antibodies for site-specific photochemical-based tagging, functionalization and immobilization in numerous applications.

1. Introduction

The humoral immune response is mediated by antibodies, acting as highly specific binders against invading pathogens. This unique binding specificity has been exploited in a vast range of antibody-based applications in biotechnology and biomedicine. The ability to engineer antibodies to meet the biophysical demands required by these applications, such as specificity, affinity, and stability [1,2] is critical. Adopting recombinant antibody libraries, representing large, renewable sources of high-performing binders [3,4], will open up new avenues for generating, designing, and tailoring binders with such desired properties [5–7].

Engineering antibodies with improved properties, such as stability, have traditionally been accomplished by mutating single or combinations of key amino acid residues [8–10], limiting the building blocks to the standard 20 amino acids. By generating novel transfer RNA (tRNA)/aminoacyl-tRNA synthetase pairs, Schultz et al. have successfully expanded the genetic code, and made it possible to genetically encode for more than 70 unnatural amino acids (UAAs) with novel and diverse physical, chemical, or biological properties in *Escherichia coli* (*E. coli*), yeast, and mammalian cells [11,12]. These novel building blocks include fluorescent, glycosylated, metal-ion-binding, and redox-active amino acids, as well as amino acids with unique chemical and photochemical reactivity [13,14], for review see [15], providing a powerful method for generating proteins with novel and/or enhanced properties. In the case of antibodies, UAA has so far been introduced to full length IgG and/or fragment antigen-binding (Fab) thereof, to generate e.g. dimeric antibodies [16], multimeric antibodies [17], antibody–drug conjugates [18], and antibody–DNA conjugates [19].

Introducing photoreactive groups in antibodies (proteins) could provide new means for tagging, functionalization, and/or surface immobilization [20–23]. To date, this has mainly been accomplished using photonic activation of accessible disulfide bridges, as illustrated for Fab antibody fragments [20,21,23]. An alternative route could be to use UAAs instead, such as the photo cross-linker *p*-benzoyl-L-phenylalanine

Abbreviations: β-CD, β-cyclodextrin; *p*Bpa, *p*-benzoyl-L-phenylalanine; Fab, fragment antigen-binding; FW-1, framework-1; MW, molecular weight; scFv, single-chain Fv variable; T_m, melting temperature; tRNA, transfer RNA; UAA, unnatural amino acids; RT, room temperature; VEGF, endothelial growth factor; VH, variable domain oft he heavy chain; VL, variable domain of the light chain

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(*p*Bpa), which bears the photoreactive benzophenone moiety [11,13,14]. The ketyl group of *p*Bpa typically reacts with C – H bonds upon excitation of near-UV light (350–360 nm) resulting in the formation of an intramolecular C – C covalent bond between reacted *p*Bpa and target molecule. Based on this, the dock'n'flash technology was recently developed providing the means to specifically and covalently photocouple *p*Bpa mutated proteins with site-specific control based on a bioaffinity mechanism [24]. In more detail, the authors successfully exploited the affinity docking of the *p*Bpa group at the protein surface into a β-cyclodextrin (β-CD) host, which were then covalently coupled in a light-dependent reaction with non-denaturing UVA, using cutinase (*Fusarium Solani Pisi* cutinase) as model protein.

In this proof-of-concept study, we have adopted the dock'n'flash technology and for the first time introduced the photoreactive UAA *p*Bpa residue into a single-chain Fv variable (scFv) antibody, selected from a human recombinant antibody phage display library, engineered around a single, constant framework (V_H3-23–V_λ1-47) [3]. The results showed that the *p*Bpa mutated scFv could be expressed with retained structural and functional properties, including binding affinity, and that the mutant could be site-specifically photocoupled to β-CD in both solution and on-chip (array immobilized). Hence, this opens up novel routes for tailoring scFv antibodies for photochemical-based tagging, functionalization, and immobilization in a wide range of applications, exemplified by, but not limited to, antibody-based microarrays [5,25].

2. Material and methods

2.1. Antibodies

A human recombinant wild-type scFv antibody, denoted a-C1q-wt, directed against the human complement protein C1q, was selected from an in-house designed phage display library, as previously described [3]. The a-C1q-wt antibody was selected as a model antibody, since it has been demonstrated to display high specificity, affinity (K_d of 2×10^{-10} M), stability, and on-chip microarray functionality [26–28]. In a similar manner, an endothelial growth factor (VEGF) specific scFv antibody, denoted a-VEGF, was selected and used as negative control.

2.2. Site-directed mutagenesis

The position of the *p*Bpa (Bachem, Bubendorf, Switzerland) mutation was chosen according to the following three criteria: 1) Far from the complementarity determining regions (CDR) in order to minimize any interference with antigen binding. 2) Within an exposed loop structure in order to maximize the access to *p*Bpa and to minimize the risk of disturbing the structure of the antibody. 3) The first base after the TAG codon should be an adenine (preferred) or guanine, to minimize the risk of premature termination of the recombinant protein [29]. To this end, a *p*Bpa mutation site was defined in the variable domain of the light chain (VL), Q17, of a-C1q-wt.

Gene encoding *p*Bpa mutated a-C1q-wt, denoted a-C1q-mutant, was generated using QuikChange®Site Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) following the instructions provided by the manufacturer. A complementary forward (5′ TCT GGG ACC CCC GGG **TAG** AGG GTC ACC ATC TCT 3′) and reverse (5′AGA GAT GGT GAC CCT **CTA** CCC GGG GGT CCC AGA 3′) substitution mutation primer pair was designed, and ordered from Eurofins MWG (Edersberg, Germany). The mutated gene construct carried in the pFab5C.his vector was then transformed into competent *E. coli* XL1-Blue competent cells. Glycerol stocks were generated and stored at −80 °C until further use. Mutagenesis was verified by DNA sequencing (Eurofins MWG Operon, Ebersberg, Germany).

2.3. Co-transformation

The a-C1q-mutant-coding plasmid was co-transformed with a supplementary plasmid, pSUP*p*Bpa-6TRN plasmid encoding the *p*Bpa tRNA/aminoacyl tRNA synthetase pair, a gift from Dr. Peter Schultz, Scripps Institute, La Jolla, CA, USA, into the production host TOP10F′ *E. coli*. Successful clones were re-sequenced (EuroFins MWG Operon) and stored as glycerol stocks at −80 °C until further use.

2.4. Antibody production

The a-C1q-wt, a-VEGF (100 μg/ml ampicillin), and a-C1q-mutant (100 μg/ml ampicillin and 25 μg/ml chloramphenicol) scFv antibodies were produced in *E. coli* (100 ml cultures). In the case of a-C1q-mutant, the growth medium (2xYT) was supplemented with 1 mM *p*Bpa [29]. Due to the light sensitivity of *p*Bpa all handling of *p*Bpa-containing preparations were performed in laboratory areas without windows and lighting. The produced scFvs were purified from either supernatant or periplasmic preparations using affinity chromatography on Ni^{2+}-NTA agarose gel (Qiagen, Hilden, Germany). Bound molecules were eluted with 1 ml 250 mM imidazole, extensively dialyzed against PBS, and stored at 4 °C, until further use. The protein concentration was determined by measuring the absorbance at 280 nm using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, USA). The degree of purity and integrity of the scFvs was evaluated using 10% SDS-PAGE (Invitrogen, Carlsbad, CA, USA) and Western-blot. In the latter case, the scFv antibodies were visualized using mouse α-MYC antibody (Zymed, San Francisco, CA, USA) and horseradish peroxidase conjugated rabbit-anti-mouse immunoglobulin (Dako Denmark A/S, Glostrup, Denmark), and developed in a KODAK X-OMAT 1000 processor (Kodak Nordic AB, Upplands Väsby, Sweden) with Amersham Hyperfilm™ ECL (GE Healthcare).

2.5. MALDI-TOF analysis

The antibody preparations were cleaned using $ZipTip_{C4}$ Pipette Tips (Millipore, Billerica, MA, USA) before adding the matrix. The tips were equilibrated by washing with 50% (*v*/*v*) acetonitrile (ACN) followed by 0.1% (*v*/*v*) TFA. Bound proteins were washed with 0.1% (*v*/*v*) TFA, and subsequently eluted with 70% (*v*/*v*) ACN 0.1% (*v*/*v*) TFA. The eluates were mixed 1:1 with matrix (10 mg/ml cinnamic acid, 40% (*v*/*v*) ACN, 0.06% (*v*/*v*) TFA) and then manually dispensed as double droplets (1 μl/drop) onto a solid support. The first droplet was allowed to dry before spotting the second. Due to problems purifying the a-C1q-mutant-CD with $ZipTip_{C4}$ tips, solutions were directly mixed with the above-mentioned matrix and spotted. Dried samples were analyzed using MALDI micro MX ™ (Waters, Milford, MA, USA) and MassLynx™ Software (Waters).

2.6. Circular dichroism

The secondary structure and thermostability of the scFv antibodies were examined using circular dichroism (CD), (J-720 Spectropolarimeter (Jasco, Eaton, MD, USA)). The scFvs were dialyzed (MWCO 12-14.000) against PBS for 1–3 days to remove sodium azide. The samples were scanned at 20 °C in the far UV, 250–200 nm. A temperature scan from 20 °C to 95 °C with a thermal ramp of 1 °C/min was also performed, and change in molar ellipticity at 218 nm was recorded, with two data points per minute. Thermal unfolding scans were analyzed assuming a two-state equilibrium between native and denatured state, and the mid-point of the transition, T_m-value (melting temperature), was estimated.

2.7. Structural modeling

A structural homology model was generated for a-C1q-wt using the Web Antibody Modelling server following the standard guidelines (http://antibody.bath.ac.uk). The structure was examined and visualized, using PyMOL Molecular Graphics System (Schrödinger, LCC). A structural model for a-C1q-mutant was generated in PyMOL by exchanging Gln17 with *p*Bpa, and by using a database for non-natural amino acid sidechain rotamers (http://swisssidechain.ch. ID for *p*Bpa: PBF). A structural model for the inclusion complex of a-C1q-mutant–β-CD was generated by manual docking in PyMOL.

2.8. Antigen and serum samples

Purified C1q was purchased from ElectraBox Diagnostica (Tyresö, Sweden). Purified C3 was obtained from Sigma (St. Louis, MO, USA). Two well-characterized (de-identified) human serum samples, including a large pool of normal human sera and a C1q complement-deficient serum samples, i.e. a well-characterized sample with significantly reduced C1q level, were used as control samples (Skåne University Hospital, Lund, Sweden).

2.9. Labeling of antigen and serum samples

Pure C1q was labeled with Alexa-647 (Molecular Probes, Eugene, OR, USA) at a molar ratio of 10:1 dye:protein in 40% (*v*/*v*) glycerol in PBS, and incubated on ice for 5 h, with careful mixing every 20 min. Next, labeled C1q was extensively dialysed against 40% (*v*/*v*) glycerol in PBS, aliquoted, and stored at −80 °C until further use.

The serum samples (about 90 mg/ml) were biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) according to previously optimized labeling protocol for serum proteome [26,28]. Briefly, the serum samples were labeled at a molar ratio of biotin: protein (15:1), and unreacted biotin was removed by extensive dialysis against PBS (pH 7.4). Finally, the samples were aliquoted and stored at −20 °C until further use.

2.10. Generation of β-CD functionalized slides

The 1st generation of β-CD functionalized slides was generated. Quartz slides (25 × 76 × 1 mm) were purchased from SPI supplies (Wester Chester, USA). β-CD was obtained from Wacker Chemie AG (Munich, Germany) and dried at 110 °C in vacuum for 24 h prior to use.

Prior to silanization the quartz slides were cleaned with a solution of HCl/MeOH (1:1) for 30 min, followed by 30 min in H_2SO_4 and then rinsed with milli-Q. In order to increase the number of surface silanols, the slides were exposed to a 6:1:1 solution of milli-Q:HCl:H_2O_2 for 10 min at 80 °C, extensively washed with milli-Q, dried under a mild stream of nitrogen and finally dried for 4 h at 110 °C in a vacuum oven. For silanization, the slides were covered with 0.5% 3-glycidyloxypropyl-trimethoxysilane in dry toluene and left to react overnight at room temperature (RT) under a nitrogen atmosphere. After silanization, the slides were rinsed extensively with toluene and EtOH, dried under a mild stream of nitrogen and if not used immediately, kept under argon until further use.

The sodium salt of β-CD was prepared by adding 140 mg (40 mM) of oil-free NaH and 100 μl *tert*-butyl alcohol to a pre-dried 250 ml reaction flask with 1 g dry β-CD (6 mM) in 150 ml dry N,N-dimethylformamide (DMF) under stirring and nitrogen atmosphere for 0.5 h. Next, the solution was filtered under nitrogen atmosphere to remove excess NaH. The solution containing the β-CD sodium salt was then transferred to a pre-dried 250 ml reaction flask, heated to 80 °C and then transferred to a preheated reaction dish holding the epoxy functionalized quartz slides. The grafting reaction was allowed to proceed overnight at 80 °C under nitrogen atmosphere and was then washed with DMF, toluene, EtOH and milli-Q, in this order and finally dried under mild stream of nitrogen.

2.11. Antibody microarray—black polymer Maxisorb slides

Antibody microarrays were produced by dispensing single droplets (about 300 pl) of a-C1q-wt, a-C1q-mutant (±β-CD), a-VEGF (negative control), and PBS (negative control) onto black polymer Maxisorb slides (Nunc A/S, Roskilde, Denmark) using the non-contact printer SciFLEXARRAYER S11 (Scienion AG, Berlin, Germany) [26,28]. The spotting reagents were arrayed in eight replicates per subarray and individual subarrays were created using a hydrophobic pen (Dako Denmark A/S). All incubation steps were performed at RT in a humidity chamber, protected from light. First, the arrays were blocked with 5% (*w*/*v*) fat-free milk powder (Semper AB, Sundbyberg, Sweden) in PBS o/n (PBS-M). The arrays were washed three times with 0.05% (*v*/*v*) Tween-20 in PBS (PBS-T) and incubated with biotinylated serum sample or directly labeled C1q (0.01 to 100 nM) for 1 h. All serum samples were diluted 1:10 (resulting in a total serum dilution of 1:450, corresponding to about 0.2 mg/ml) in 1% (*w*/*v*) fat free milk powder and 1% (*v*/*v*) Tween-20 in PBS (PBS-MT). Next, the arrays were washed three times with PBS-T. Any subarrays incubated with biotinylated serum samples were incubated with 1 μg/ml Alexa-647-conjugated streptavidin (Invitrogen) diluted in PBS-MT for 1 h. Finally, the arrays were washed three times with PBS-T and one time with PBS and directly dried under a stream of nitrogen gas and immediately scanned.

2.12. Antibody microarray—β-CD-functionalized slides

Antibody microarrays (a-C1q-wt, a-C1q-mutant, and a-VEGF (negative control) were prepared by manually dispensing 1 to 5 μl on pre-chilled (4 °C) β-CD functionalized slides and incubated for 1 h at 4 °C to allow for non-covalent inclusion complex formation (between *p*Bpa mutated protein and surface immobilized β-CD). Photo-immobilization (covalent coupling) (dock'n'flash technology [24]) was achieved by placing the slides directly on the pre-chilled 10 W low-pressure mercury arc UVA lamp (USHIO, Cypress, CA) and illuminating them at 365 nm for 15 min at 4 °C. Unbound proteins were removed by washing with 0.5% (*w*/*v*) CHAPS in PBS for 4 h at 4 °C. The microarrays were then washed once with PBS-M, and then blocked overnight in PBS-M. Next, the slides were handled as described under 2.11.

2.13. Reverse antibody microarrays

Reversed antibody microarrays were produced by dispensing single droplets (about 300 pl) of 0.1 mg/ml pure antigen (C1q and C3) onto black polymer Maxisorb slides (Nunc) using the non-contact printer SciFLEXARRAYER S11 (Scienion). The antigens were spotted in eight replicates per slide. Individual subarrays were created using a hydrophobic pen (Dako Denmark A/S). All incubation steps were performed at RT for 1 h in a humidity chamber protected from light. The microarrays were blocked with PBS-M, washed with PBS-MT, and subsequently incubated with 1 μg/ml scFv (a-C1q-wt and a-C1q-mutant). After washing, the arrays were incubated with 0.2 μg/ml α-His mouse IgG in PBS-MT for 1 h. Subsequently, the arrays were incubated with 0.2 μg/ml Cy5-conjugated AffiniPure F(ab′) goat-anti-mouse IgG (Fcγ fragment specificity) in PBS-MT. After a final washing with PBS-T, the arrays were rinsed with pure PBS, dried under a stream of nitrogen gas and immediately scanned.

2.14. Scanning of antibody microarrays

All antibody arrays were scanned using a confocal microarray scanner (Perkin-Elmer Life & Analytical Sciences) at 5 or 10 μm resolution, using three different scanning settings (70% PMT gain and 70% laser

power (70/70)), 80/80, 100/100. The signal intensity of each spot was quantified using the fixed circle method by ScanArray Express software V4.0 (Perkin Elmer Life & Analytical Sciences). The local background was subtracted and to compensate for possible local defects, the two highest and the two lowest signal intensities were automatically excluded, and each data point represents the mean value of the remaining four replicates. For spots displaying saturated signal, values from lower scanning settings were chosen. Only data generated using identical scanner settings were compared.

2.15. Photo-cross-linking of a-C1q-mutant to β-CD in solution

Photo-cross-linking of a-C1q-mutant and β-CD in solution was performed by mixing the antibodies (1:1) with 2 to 5 mM β-CD in PBS in glass vials. The mixture was incubated for 1–2 h at 4 °C, and subsequently illuminated for 20 min at 365 nM at 4 °C with a 10 W low-pressure mercury arc UVA lamp (USHIO). The reaction mixtures were stored at 4 °C prior to use. The non-mutated wild type antibodies, a-C1q-wt and a-VEGF, were used as negative controls.

2.16. Binding affinity

The binding affinity, in terms of apparent K_d, was determined for a-C1q-wt and a-C1q-mutant using antibody microarrays on black polymer Maxisorb slides (see Section 2.11). The antibodies were dispensed in 24 replicates, and visualized by adding directly labeled C1q (see Section 2.9). First, the amount of antibody was titrated (0.025 to 0.2 mg/ml) in order to define the optimal concentration (0.09 mg/ml) of antibody to reach B_{max} (the maximum specific binding) without saturating the detector. Next, serial dilutions ($n = 13$, ranging from 0.1 to 100 nM) of directly labeled antigen were analyzed on the arrays (see Section 2.11). Non-linear regression analysis (one site, specific binding) of the data was performed using GraphPad Prism 6 (GraphPad Software Inc, San Diego, USA) to determine B_{max} and then K_d.

3. Results

In order to explore whether recombinant scFv antibodies could be tailored to display photoreactive properties compatible with the dock'n'flash technology, a human recombinant scFv antibody (a-C1q-wt) was mutated to carry the photoreactive UAA *p*Bpa (Fig. 1A). The resulting mutant, a-C1q-mutant, was characterized and its affinity interaction with β-CD, converted into a covalent light-dependent interaction, was examined both in solution and on-chip.

3.1. Design of pBpa mutated scFv

Site-directed mutagenesis was used to introduce the UAA *p*Bpa into the constant framework of the model scFv. Three key criteria were considered when selecting the site of mutation. The site of mutation should be located in an exposed loop structure far away from the antigen binding site to minimize any impact on the structural and functional properties of the antibody, as well as to optimize the accessibility of *p*Bpa (Fig. 1). In addition, the first base following the amber codon (TAG) should be an adenine (preferred) or guanine (Fig. 2A) in order to promote expression. Based on structural and sequence analysis, Q17, located in framework 1 (FW-1) of VL, was identified as a potentially suitable point of mutation (Figs. 1B and 2A).

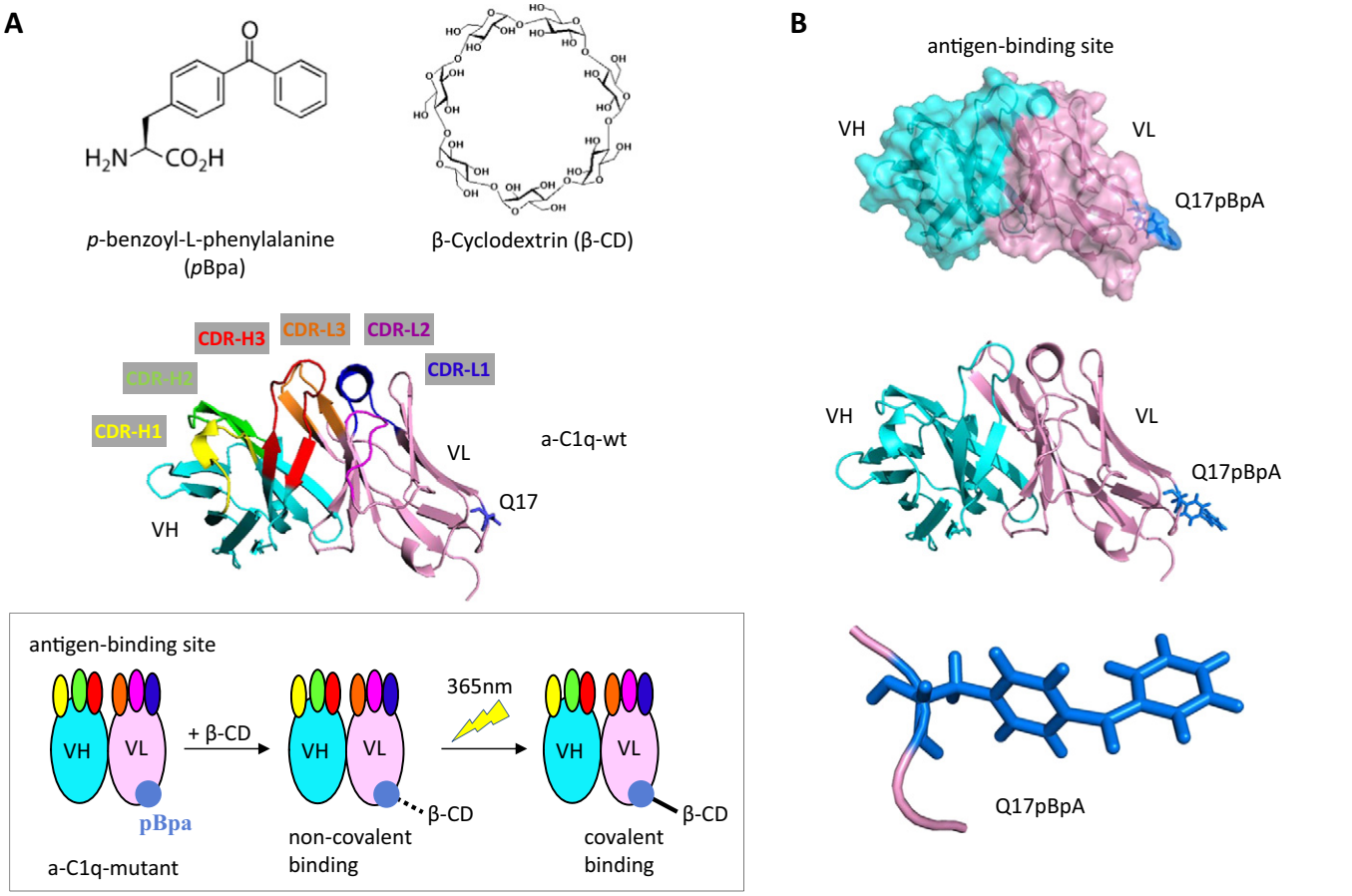

Fig. 1. Tailoring of a human recombinant scFv antibody to carry a photoreactive UAA, compatible with the dock'n'flash technology. (A) Structure of the photoreactive UAA *p*Bpa and its (host–guest) interaction partner, β-CD, as well as of the scFv antibody (side view). The site of mutation, Q17, located in VL is indicated. (B) Structural homology model of the *p*Bpa mutated scFv (side view), highlighting the Q17*p*Bpa mutation.

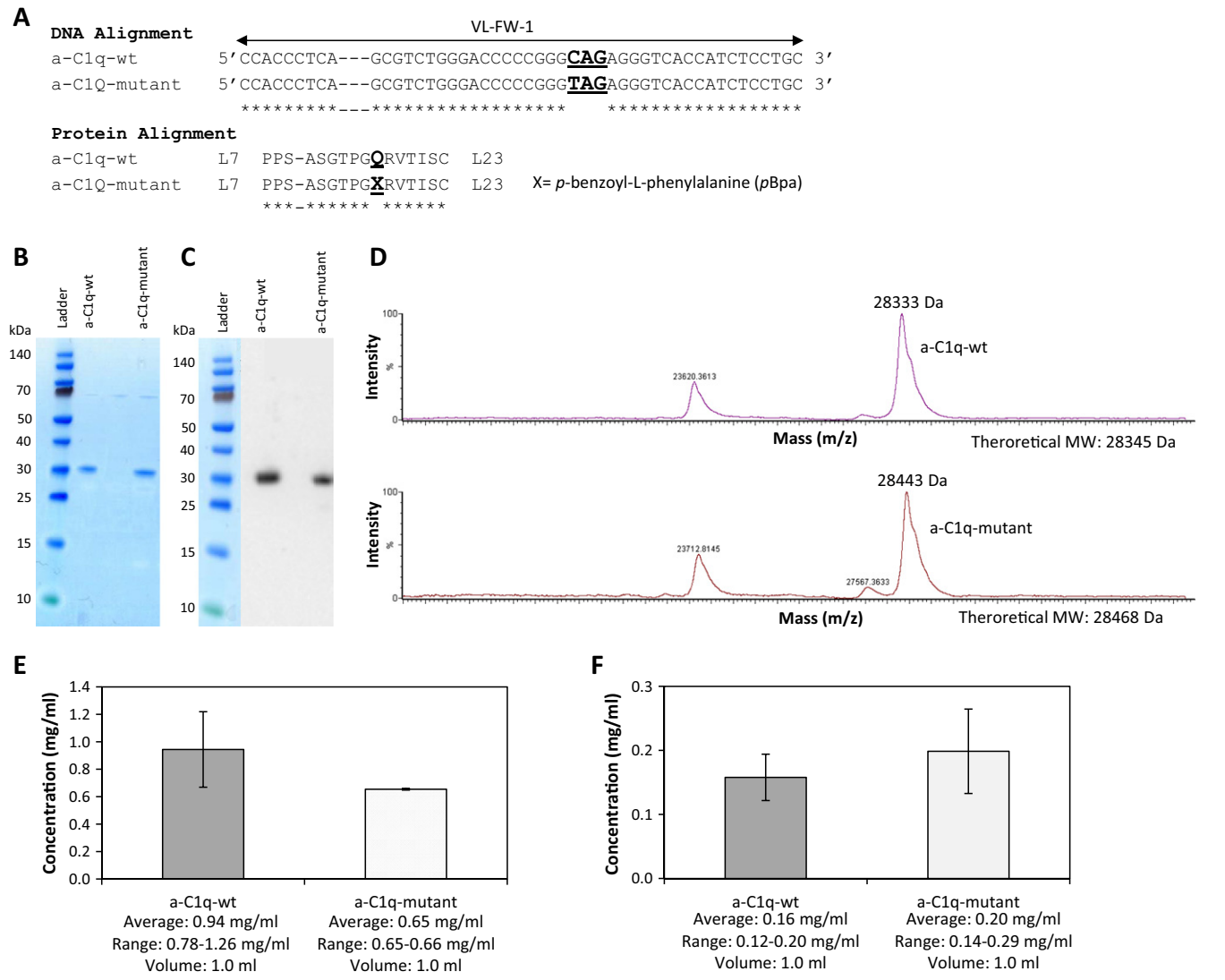

Fig. 2. Sequence analysis and protein expression of *p*Bpa mutated scFv. (A) DNA sequences and corresponding protein sequences of a-C1q-wt and a-C1q-mutant. TAG represents the amber codon used to encode for *p*Bpa. (B) SDS-PAGE and (C) western-blot analysis of a-C1q-wt and a-C1q-mutant. (D) MALDI-TOF MS analysis of a-C1q-wt and a-C1q-mutant. (E) Protein expression analysis (3 batches in Top10F′ *E. coli*) of a-C1q-wt vs. a-C1q-mutant purified from periplasmic preparations. (F) Protein expression analysis (4 batches in Top10F′ *E. coli*) of a-C1q-wt vs. a-C1q-purified from supernatant preparations.

3.2. Sequence analysis and protein expression of pBpa mutated scFv

Sequence analysis confirmed the successful introduction of the amber codon, TAG, encoding for *p*Bpa in position 17 in FW-1 of VL (Fig. 2A). Providing the Top10F′ *E. coli* protein machinery with the matching unnatural tRNA/aminoacyl-tRNA synthetase pair and *p*Bpa, the SDS-PAGE (Fig. 2B) and Western blot analysis (Fig. 2C) showed that a dominant protein product with an apparent molecular weight (MW) of about 30 kDa was produced for both a-C1q-wt (theoretical MW of 28,345 Da) and a-C1q-mutant (theoretical MW of 28,468 Da) (Fig. 2B). Further, the introduction of *p*Bpa was also confirmed using MALDI-TOF MS, demonstrating that the molecular weight of the dominant antibody peak (28,333 vs. 28,443 Da) had increased about 110 Da upon introduction of the Q17*p*Bpa mutation (theoretical MW increase 123 Da) (Fig. 2D).

Next, analysis of protein expression (3 to 4 independent batches of 100 ml cultures) showed that *p*Bpa mutated scFv could be expressed in Top10F′ *E. coli*, and be purified from both periplasmic (Fig. 2E) and supernatant (Fig. 2F) preparations using affinity chromatography on Ni^{2+}-NTA. Compared to the wild-type scFv, the average production yields were lower (69%) based on periplasmic preparations (0.65 mg/ml vs. 0.94 mg/ml; 1.0 ml fractions), but more similar when based on supernatant preparations (0.20 mg/ml vs. 0.16 mg/ml; 1.0 ml fractions). Focusing on the periplasmic preparations, which gave the apparently best production yield, three additional independent production batches were produced. Again, the data showed that the yield of the *p*Bpa mutated scFv was lower (in average 73%) than that of the wild-type antibody.

3.3. Structural analysis of pBpa mutated scFv

In an attempt to investigate the structural impact of the *p*Bpa mutation on the scFv antibody structure, circular dichroism (CD) measurements were performed on both the a-C1q-wt and a-C1q-mutant. First, the overall secondary structure was compared by recording the CD wavelength spectra (200–250 nm) at 20 °C (Fig. 3A). Similar curves were observed for both mutated and wild-type scFv, with typical shape and negative maximum molar ellipticity at about 218 nm, characteristic for the dominant anti-parallel β-sheet structure found in native

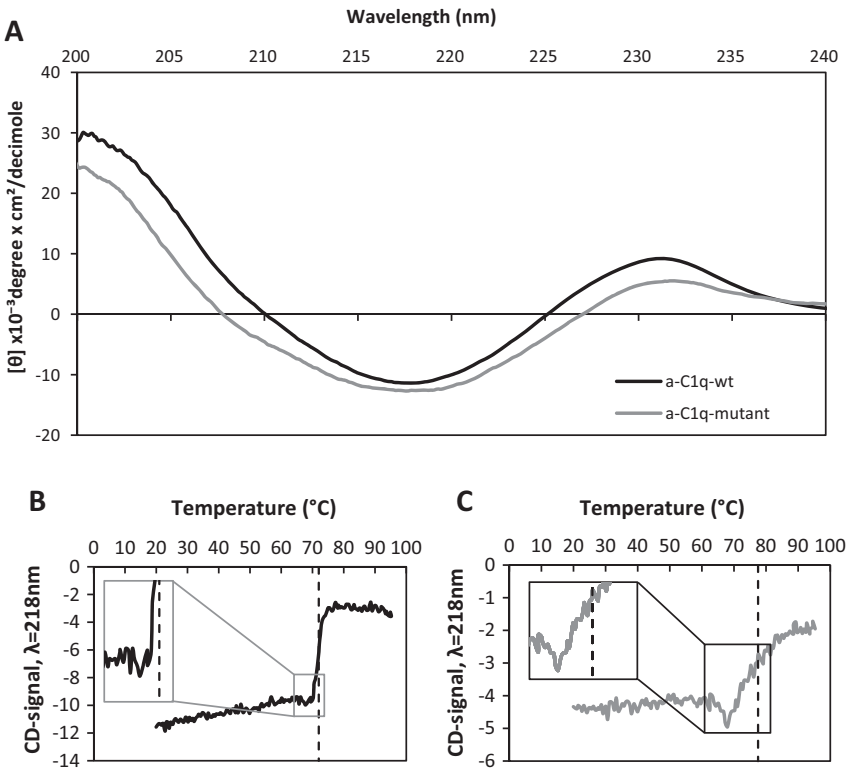

Fig. 3. Structural analysis of *p*Bpa mutated scFv. (A) CD wavelength scans for a-C1q-wt (0.42 mg/ml) and a-C1q-mutant (0.14 mg/ml) at 20 °C. The data has been corrected for the differences in protein concentration in order to enable a direct comparison. (B) Thermal denaturation scans at 218 nm for a-C1q-wt (B) and a-C1q-mutant (C). The dashed line indicates the T_m-value.

scFv. Hence, the data implied that the secondary structure was maintained in the *p*Bpa mutated scFv.

Next, the structural thermo-stability of scFv in solution was determined for a-C1q-wt (Fig. 3B) and a-C1q-mutant (Fig. 3C), using thermal scans from 20 to 95 °C monitored at 218 nm. In both cases, a single apparent cooperative unfolding transition was observed, although with different characteristics. For a-C1q-wt, the transition from native to denatured state was sharp starting at 67 °C and ending at 75 °C, whereas for a-C1q-mutant the transition endpoints were at 67 °C and 89 °C. In addition, a more noticeable increase in ellipticity characterized by a "drop" in the scan at about 67 °C was observed for a-C1q-mutant at the start of the native to denature state transition (cfs. Fig. 3B and C). After fitting the data with a two-state equilibrium model, T_m-values were extracted and showed to be higher for a-C1q-mutant than for a-C1q-wt, 77 °C vs. 72 °C (cfs. Fig. 3B and C). The melting curve for the a-C1q-mutant was less well-defined, due to a lower protein concentration and a lack of data points in the upper range of temperatures, which ended in a less reliable curve fitting and T_m-value estimation. However, on the basis of T_m-values, the results indicated that the introduction of the UAA *p*Bpa marginally improved the structural stability in solution of the scFv antibody, but also modified its unfolding landscape.

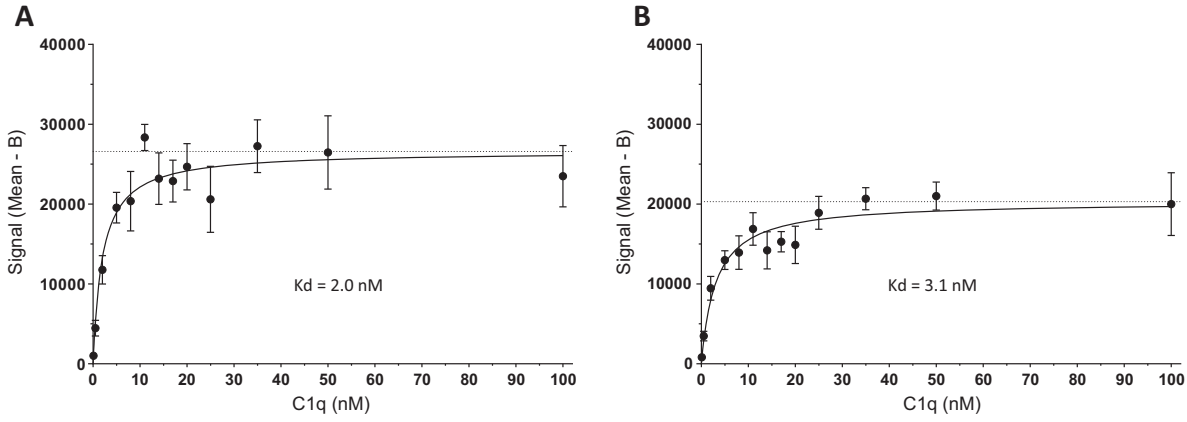

Fig. 4. Binding affinity (K_d) of (A) wild-type scFv and (B) *p*Bpa mutated scFv. The binding affinity was determined by analyzing serial dilutions of directly labeled antigen on antibody microarrays.

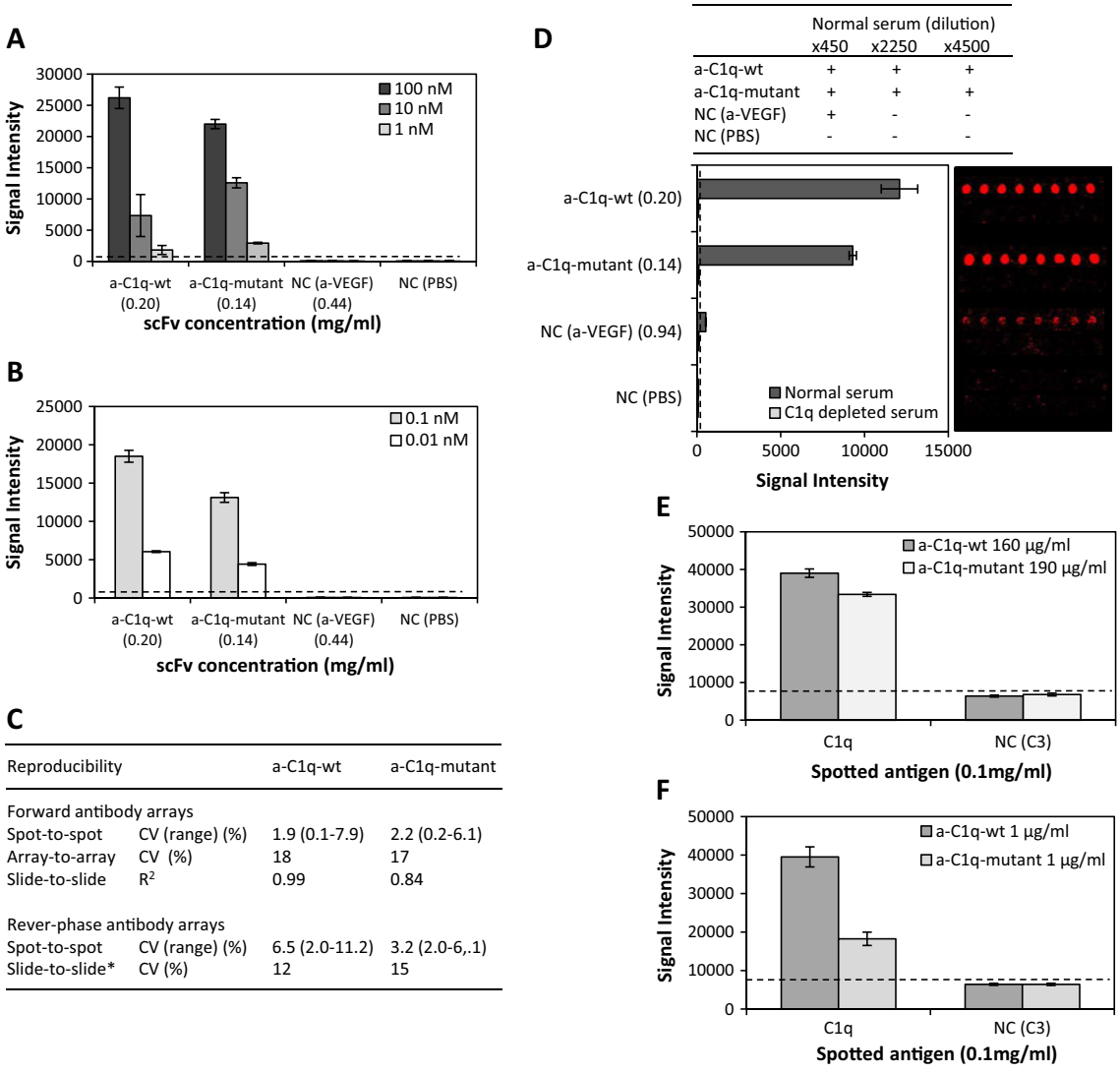

Fig. 5. Functional activity of *p*Bpa mutated scFv, purified from both periplasmic and supernatant preparations (similar results were obtained irrespective of batc used). (A) Antibody microarray analysis of the on-chip activity of a-C1q-wt vs. a-C1q-mutant, targeting serial dilution of pure, labeled C1q. (B) Antibody microarray analysis of the on-chip activity of a-C1q-wt vs. a-C1q-mutant, targeting serial dilution of pure, labeled C1q. (C) Reproducibility of the (forward) antibody microarray analysis, including spot-to-spot (based on 8 replicates/subarray, and 9 subarrays), array-to-array (3 subarrays/slide, 8 spots/subarray, 1 slide), and slide-to-slide (2 subarrays/slide, 8 spots/subarray, 3 slides). Reproducibility of the reverse-phase antibody microarray analysis, including spot-to-spot (based on 8 replicates/subarrays, and 8 subarrays), and slide-to-slide (2 subarrays/slide, 8 spots/subarray, 2 slides, produced on different days). (D) Antibody microarray analysis of the on-chip activity and specificity of a-C1q-wt and a-C1q-mutant targeting normal and C1q-deficient crude, biotinylated human serum samples. (E) Reverse-phase antibody microarrays analysis of the functional activity in solution of a-C1q-wt vs. a-C1q-mutant, targeting pure arrayed C1q. (F) Reverse-phase antibody microarrays analysis of the functional activity in solution of a-C1q-wt vs. a-C1q-mutant, targeting pure arrayed C1q.

3.4. Binding affinity and functional activity of pBpa mutated scFv

To compare the binding affinity and functional activity of *p*Bpa mutated vs. wild-type scFv, conventional antibody microarrays (binding affinity/on-chip activity—the antibodies were spotted onto slides, and soluble antigen was applied in serial dilutions) and reverse-phase antibody microarrays, or antigen microarrays (activity in solution—the antigen was spotted onto slides, and soluble antibodies was applied), were produced and analyzed (Figs. 4 and 5).

First, the apparent binding affinity (K_d) was determined by analyzing serial dilutions of directly labeled C1q (Fig. 4). The results showed that the a-C1q-wt and a-C1q-mutant scFv antibodies displayed similar K_d values, 2.0 and 3.1 nM, respectively. Hence, the data implied that the UUA *p*Bpa could be introduced into the framework of the scFv antibody without impairing the binding affinity.

Targeting a serial dilution of pure antigen (C1q), the antibody microarray analysis showed that arrayed a-C1q-wt and a-C1q-mutant displayed similar reactivity pattern and degree of activity as reflected by the observed signal intensities (Fig. 5A and B). In both cases, the limit of detection was below 10 pM pure C1q. The technical reproducibility of the antibody array-based assay (a-C1q-wt vs. a-C1q-mutant), including spot-to-spot (CV of 1.9% vs. 2.2%), array-to-array (CV of 18% vs. 17%), and slide to-slide (R^2 of 0.99 vs. 0.84) was found to be similar (Fig. 5C). Noteworthy, similar reactivity patterns were also observed for both antibody clones targeting serial dilutions of crude biotinylated serum containing C1q (Fig. 5D), indicating that the functional activity of the *p*Bpa mutated scFv was retained. The limit of detection was below ×4500 dilution of crude serum (< ~90 pM C1q). Most importantly, no signals were observed for either of the two antibody clones targeting a C1q deficient serum sample, indicating high and retained specificity. The fact that one of the negative controls, a-VEGF, gave a

positive signal (×450 dilution only) could be explained by the fact that the serum sample contained VEGF.

In the case of the reverse-phase antibody microarrays, targeting pure arrayed C1q, lower signals intensities were observed for the a-C1q-mutant vs. a-C1q-wt analyzed at two different concentrations (Fig. 5E and F), indicating that the *p*Bpa mutated scFv displayed lower functional activity in solution. The technical reproducibility of the reverse-phase antibody arrays (a-C1q-wt vs. a-C1q-mutant), including spot-to-spot (CV of 6.5% vs. 3.2%) and slide-to-slide (different days) (CV of 12% vs. 15%) was found to be in the same range (Fig. 5C).

3.5. Dock'n'flash technology—photocoupling of pBpa mutated scFv to β-CD

Finally, we investigated whether *p*Bpa mutated scFv could be photocoupled to β-CD in both solution and to β-CD functionalised quartz slides using the dock'n'flash technology (Fig. 6).

First, β-CD (MW of 1135 Da) was mixed with either a-C1q-wt or a-C1q-mutant and exposed to light at 365 nm. MALDI-TOF MS analysis showed that the wild-type scFv was, as expected, not photocoupled to β-CD (Fig. 6A). In contrast, the MS analysis showed that the MW of the *p*Bpa mutated scFv increased by 1,112 Da, confirming the site-specific photocoupling to β-CD (Fig. 6B). The yield of photocoupled antibody was apparently about 50% in the experimental conditions which were used.

The on-chip functionality of arrayed a-C1q-mutant was then determined before and after photocoupling to β-CD, targeting both pure labeled C1q (Fig. 6C and D) and crude, biotinylated serum containing C1q (Fig. 6E and F). Using the activity of wild-type scFv as a reference, the microarray data showed that the *p*Bpa mutated scFv displayed similar activity whether or not it had been coupled to β-CD, indicating that the photocoupling had no, or only minor, effects on the on-chip functionality. A structural model of β-CD is shown in Fig. 6G, outlining the hydrophobic cavity created, into which *p*Bpa will bind with moderate affinity to form an inclusion complex.

Next, the a-C1q-wt and a-C1q-mutant were manually dispensed onto the 1st generation of β-CD coated quartz slides and exposed to light at 365 nm (Fig. 6H). After washing, the slides were incubated with a crude, biotinylated serum sample containing C1q. The results showed that high signal intensities were only observed for the a-C1q-mutant antibody, while the spots with a-C1q-wt displayed signal

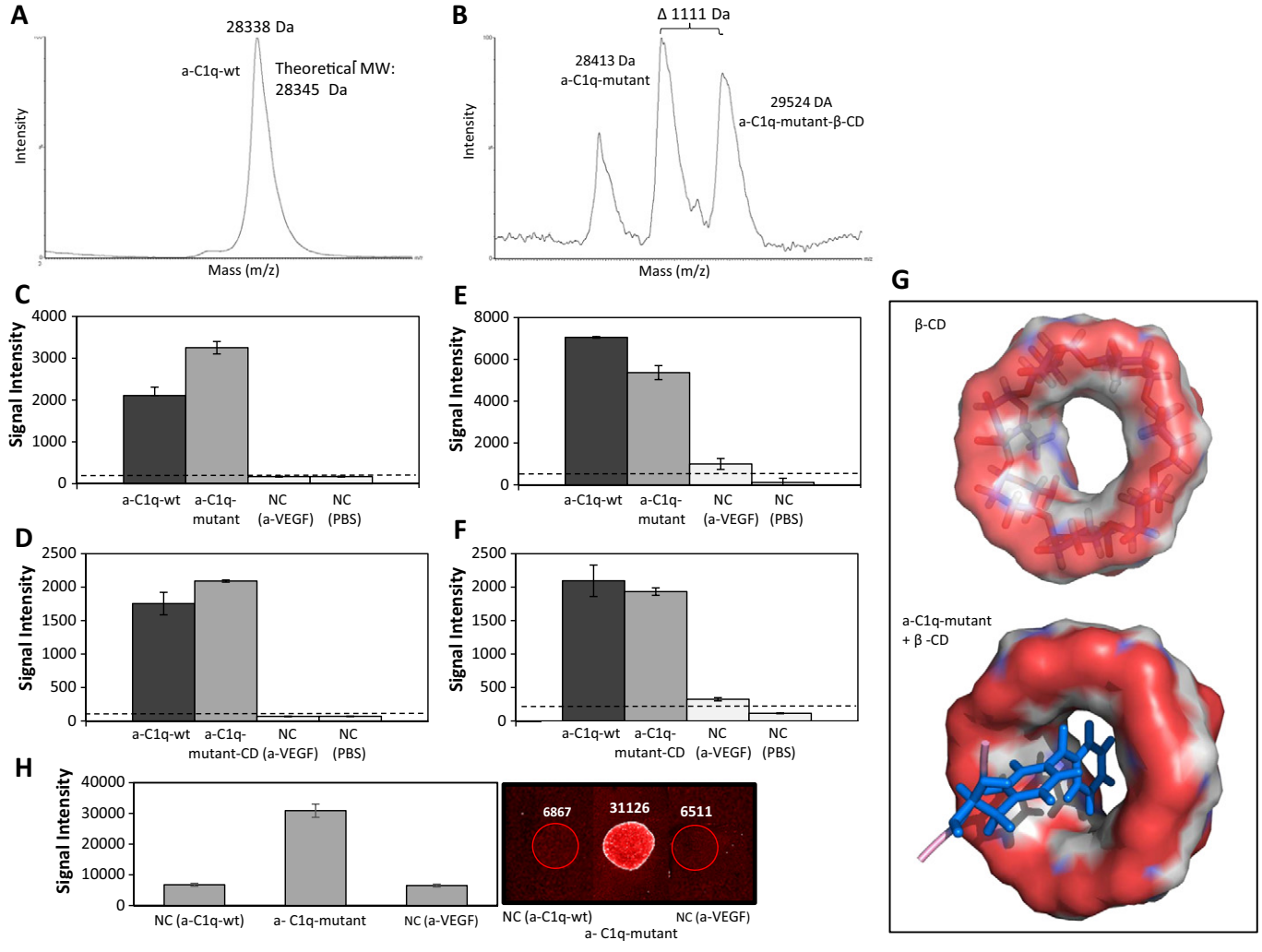

Fig. 6. Dock'n'flash technology—photocoupling of *p*Bpa mutated scFv to β-CD. (A, B) MALDI-TOF MS analysis of a-C1q-wt (A) and a-C1q-mutant (B) after incubation with β-CD and exposure to light of 365 nm. (C) Antibody microarray analysis of the on-chip activity of a-C1q-wt vs. a-C1q-mutant before photocoupling to β-CD, targeting pure, labeled C1q. (D) Antibody microarray analysis of the on-chip activity of a-C1q-wt vs. a-C1q-mutant after photocoupling to β-CD, targeting pure, labeled C1q. (E) Antibody microarray analysis of the on-chip activity of a-C1q-wt vs. a-C1q-mutant before photocoupling to β-CD, targeting crude, biotinylated serum. (F) Antibody microarray analysis of the on-chip activity of a-C1q-wt vs. a-C1q-mutant after photocoupling to β-CD, targeting crude, biotinylated serum. (G) Structural (homology) models of β-CD and in the inclusion complex of a-C1q-mutant–β-CD. In the latter case, a close up of the *p*Bpa–β-CD interaction is shown. (H) Antibody microarray analysis of the on-chip activity of a-C1q-wt vs. a-C1q-mutant after photocoupling (photo-immobilization) to β-CD functionalized quartz slides targeting crude, biotinylated serum. The spot intensity values are stated. Identical scanner and image settings were used for all three spots. Signal intensities for replicate spots/arrays are shown.

intensities similar to that of the negative control (a-VEGF), i.e. background signals. Hence, the data showed that *p*Bpa mutated scFv, but not wild-type scFv, had been successfully photocoupled to β-CD coated quartz slides using the dock'n'flash technology.

4. Discussion

In this conceptual study, we have for the first time tailored human recombinant scFv antibodies for site-specific photocoupling through the use of UAAs and dock'n'flash technology. In more detail, we have successfully introduced the photoreactive UAA *p*Bpa, and showed that the mutated scFv antibody could be expressed in *E. coli* with retained structural and functional properties, including binding affinity, and that it could be specifically photocoupled to free and surface (array) grafted β-CD after inclusion complex formation.

The 7-membered sugar ring of β-CD can be topographically represented as a toroid with the larger and smaller openings of the toroid exposing the solvent secondary and primary hydroxyl groups, respectively [30]. The cavity of β-CD will be hydrophobic, or at least considerably less hydrophilic than the aqueous environment, and thus be able to host other hydrophobic molecules. The *p*Bpa mutated scFv (guest) and β-CD (host) will form an inclusion complex with moderate affinity, and NMR analysis has indicated that the entire benzophenone ring might enter the cavity [24]. In the subsequent photocoupling step, β-CD will provide the hydrogen atoms to be abstracted. The yield of in-solution photocoupled antibody was apparently about 50% in the experimental conditions, which were used here, leaving room for improvements. Still, similar yields were observed when photocoupling cutinase in similar conditions [24], and the equilibrium could e.g. reflect that the photoirradiation was not efficient enough (re-optimized protocol warranted) and/or the affinity of the *p*Bpa–β-CD complex formation. In future efforts, the moderate affinity of the initial inclusion complex formation might be improved by substituting the secondary hydroxyl groups on the wider rim facing the protein surface in a manner to match the properties of the amino acids flanking *p*Bpa.

In comparison, light-induced immobilization of (recombinant) Fab antibody fragments to biosensor surfaces have previously been accomplished [20,21,23]. However, this approach was based on photoreduction of solvent-accessible disulfide bridges via the photoactivation of native tryptophan or tyrosine residues in full IgGs or Fab fragments. Consequently, this approach necessitated lower, more energetic and potentially damaging wavelengths (270–290 nm), and was not specific to a single amino-acid residue. In addition, disulfide photoreduction is not compatible with the structural features of scFv designs [1]. In this context, it might be of interest to note that we have designed recombinant scFv antibodies carrying a terminal cysteine-tag available for immobilization and tagging etc, but the production yields of these novel scFv designs were unsatisfactory (Wingren et al., unpublished observations). Furthermore, while traditional epitope tags, such as the His_6-tag carried by our scFv antibodies, might be explored to specifically functionalize an antibody, the His_6-tag would only provide low-/medium-affinity binding. Covalent binding or high affinity binding is essential in order to avoid dissociation, which could impair the setup. Although direct modifications, such as biotinylation, could be utilized to provide high-affinity binding (to streptavidin), achieving site- and mono-specific biotinylation is challenging, unless UAA with pendant biotin [15] or the so-called Avi-tag (a 13 amino acid peptide tag enabling site-directed biotinylation) [31] are explored. But in any case, such an approach would not be compatible with setups targeting biotinylated samples, which is the case in many antibody microarray-based platforms, including those utilized by our group.

Tailoring the structural and functional properties of antibodies to meet the biophysical demands of various applications, exemplified but not limited to antibody-based microarrays [5,25], is critical. In this context, it should be noted that we have used in-house designed human recombinant scFv phage display libraries as probe source for our applications [3] (Persson et al., unpublished data). Noteworthy, compared to other scFv antibody libraries [4], our libraries are engineered around a single, constant framework (predominantly V_H3-23 and V_λ1-47). These variable domains, VH and VL, were carefully selected, based on the excellent structural and functional properties displayed by the domains, both stand-alone as well as when paired together [1]. This library design is a key feature, since all the antibody clones ($>1 \times 10^{10}$) are structurally identical, differing only in their CDR-regions, i.e. the antigen-binding sites. In turn, this means that i) the antibodies will display biophysical properties as similar as possible, and that ii) a re-design (optimization) (e.g. point mutation) of the scaffold can simultaneously be pin-pointed for and introduced to all library members [5]. Through traditional antibody engineering approaches, we have also identified novel framework point mutations that influenced the structural and functional stability of the scFv antibodies, taking the first steps toward microarray adapting the antibodies by molecular design [5] (Nordström et al., unpublished observations; Wingren et al., unpublished observations).

Expanding the genetic code of *E. co*li through the use of UAAs opens up entirely new avenues for exploring novel building blocks to tailor antibodies [16–19], and proteins in general [11–15], with the desired property, or properties, not normally found in proteins. The introduction of photoreactive groups, such as *p*Bpa, combined with a β-CD host and the dock'n'flash technology [24], paves the way for photochemical-based tagging, functionalization and immobilization in a vast range of applications. In this study, this was exemplified by immobilization to the 1st generation of in-house designed β-CD functionalized quartz slides, which in the long-term run could enable crude antibody preparations to be purified, coupled, enriched, and specifically orientated in a one-step procedure directly on the array surface (affinity-on-a-chip). Additional work will be required in order to optimize the β-CD functionalized for array-based applications. Adopting such affinity-on-a-chip approaches, recent work has shown that the performance of antibody (protein) microarrays produced using affinity directed immobilization vs. standard coupling was in many cases superior [32,33]. This type of photochemistry could also be explored to immobilize antibodies (proteins) in self-assembly array setups, i.e. array platforms where the antibodies (proteins) are expressed directly on the array in each spot location using cell-free in situ translation from preprinted DNA [34–37]. Other exciting future efforts could be to tag the antibodies with β-CD-DNA and use them as building blocks in self-addressable antibody array setups [38,39] (Wingren et al. unpublished observations). In more detail, the antibodies are then equipped with unique zipcode tags, based on e.g. DNA, and when added to the array in bulk, the antibodies will find their way on their own to their unique address (spot) on the array composed of e.g. complementary DNA. In this context, it might of interest to note that Kazane et al. have designed site-specific Fab-DNA conjugates through the use of another UAA, *p*-acetylphenylalanine, with the aim to perform specific and sensitive immune-PCR [19].

Re-designing any protein structure and introducing a mutation, using either any of the 20 standard amino acids or the +70 UAAs as building blocks, might impair the structural and functional properties of the target protein. In this study, only minor adverse effects were observed. With respect to the observed differences in structural properties, additional experiments will be required in order to explain the effects on thermal stability (T_m-value) and unfolding transition, as well as the potential implications thereof. Due to calibration issues, the molecular weights estimated using MALDI-TOF MS consistently appeared to be about 10–20 Da lower than the theoretically calculated values. Notably, the non-annotated peak (~27,500 Da) observed in the MS run of the antibody–*p*Bpa–CD complex was present in both the wild-type and *p*Bpa mutated antibody preparations, but the proportion was apparently lower. This might be influenced by the fact that the complex was analyzed at a significantly lower antibody concentration. This peak has not been observed in MS analysis of corresponding

complexes based on other antibody clones based on the same framework, why it cannot be explained by a by-product of photoreaction, like polypeptide cleavage (Wingren et al., unpublished data). In this context, it might be of interest to note that the use of wavelengths above 340 nm for photoexcitation should considerably minimize any potential photodamage to native amino acids, tryptophan in particular. In future efforts, the site of mutation will be addressed in order to minimize any potentially unfavorable effects on the protein production, structure, binding, and function, as well as optimize the accessibility of *p*Bpa for photocoupling. Adopting a rational design strategy, we will continue to explore other positions located in exposed loops of both VH and VL, as far as possible from the antigen binding-site. Positions close to the C-terminal affinity tag will also be exploited. In addition, additional antibody clones will be mutated in order to reveal and scrutinize any clone-dependent behavior.

Taken together, in this pilot study, we have demonstrated the feasibility for designing and producing recombinant scFv antibodies carrying UAA with retained structural, functional and binding properties. By introducing the photoreactive *p*Bpa group, we tailored the recombinant antibodies for site-specific photocoupling using the dock'n'flash technology. In the long-term run this will pave the way for photochemical-based tagging, functionalization, and/or immobilization in a wide range of protein-based applications.

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